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## Stable C and N isotope concentration in several tissues of the loggerhead sea turtle *Caretta caretta* from the western Mediterranean and dietary implications

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**SUMMARY:** The isotopic concentrations of carapace scutes, skin, muscle and blood of loggerhead sea turtles (*Caretta caretta*) from the Balearic Archipelago were analysed to investigate the pattern of variation between tissues and to assess the position of this species in the trophic webs of the Algerian Basin. Skin showed higher  $\delta^{13}\text{C}$  values than muscle or carapace scutes and these showed higher values than blood. Conversely, muscle showed higher  $\delta^{15}\text{N}$  values than skin, skin showed higher values than blood and blood showed higher values than carapace scutes. Dead and live sea turtles from the same habitat did not differ in the concentration of stable isotopes. However, some of the tissues of the turtles caught in drifting long-lines in the oceanic realm showed higher  $\delta^{13}\text{C}$  values than those from the turtles caught by hand or in trammel nets over the continental shelf, although they did not differ in the  $\delta^{15}\text{N}$ . Comparison of the concentration of stable isotopes in the turtles with that of other species from several areas of the Algerian Basin revealed that they consumed planktonic prey and that the trophic level of the sea turtles was higher than that of carnivorous cnidarians but lower than that of zooplanktophagous fish and crustaceans.

**Keywords:** tissues, stable isotopes, sea turtle, trophic level, feeding ecology, carbon, nitrogen.

**RESUMEN:** CONCENTRACIÓN DE ISÓTOPOS ESTABLES DE C Y N EN VARIOS TEJIDOS DE LA TORTUGA BOBA *CARETTA CARETTA* DEL MEDITERRÁNEO OCCIDENTAL E IMPLICACIONES SOBRE LA DIETA. – La concentración isotópica de escudos del caparazón, piel, músculo y sangre de tortuga boba (*Caretta caretta*) fueron analizados para investigar el patrón de variación entre tejidos y para evaluar la posición de esta especie en las redes tróficas de la cuenca Argelina. La piel presentaba valores más altos de  $\delta^{13}\text{C}$  que el músculo o los escudos del caparazón y éstos presentaban valores más altos que la sangre. En cambio, el músculo presentaba valores más altos de  $\delta^{15}\text{N}$  que la piel, ésta valores más altos que la sangre y ésta valores más altos que los escudos del caparazón. Las tortugas muertas y las vivas del mismo hábitat no diferían en la concentración de isótopos estables. Sin embargo, alguno de los tejidos de las tortugas capturadas mediante palangre de superficie en el medio oceánico presentaban valores más altos de  $\delta^{13}\text{C}$  que los de las tortugas capturadas a mano o mediante trasmallo en la plataforma continental, aunque no diferían en el  $\delta^{15}\text{N}$ . La comparación de la concentración de isótopos estables de tortuga con la de otras especies de varias áreas de la cuenca Argelina reveló que consumían presas planctónicas y que el nivel trófico de las tortugas era superior que el de los cnidarios carnívoros pero inferior que el de peces y crustáceos zooplanctófagos.

**Palabras clave:** tejidos, isótopo estable, tortuga marina, nivel trófico, ecología trófica, carbono, nitrógeno.

### INTRODUCTION

Traditionally the diet of marine vertebrates has been investigated by stomach content analysis.

However, results are sometimes inconsistent, which may be due to at least two factors. Firstly, the prevalence of prey with hard parts is likely to be overestimated, because it is difficult to detect soft prey,

such as cnidarians and ctenophores (Plotkin *et al.*, 1993; Michener and Schell, 1994). Secondly, access to live, free-ranging specimens of scarce marine species such as sea turtles and some marine mammals is limited, which has forced most studies to rely on stranded animals. Reasons for stranding may vary. However, in diseased individuals, feeding behaviour in the period immediately preceding stranding may have been altered by weakness and include unusual prey that can bias the results provided by stomach content studies. In addition, as many stranded specimens are killed by fishing activities, diet may spuriously appear to be dominated by bait species which would otherwise only be marginal to the normal diet.

Stable isotope studies offer a powerful and practical alternative to stomach content analyses. The analysis of stable carbon and nitrogen isotopes enables feeding habits to be investigated using tiny samples of tissue collected either from live or dead animals (Michener and Schell, 1994; Kelly, 2000; Post, 2002). This technique is based on the fact that the carbon and nitrogen in an animal's body come directly from the food it consumes and hence the isotopic composition of an organism reflects that of its food resources. However, none of the sources of bias that may hinder stomach content analysis of stranded animals affect stable isotope analysis in poikilothermic animals, as the concentration of stable isotopes in the tissues of those organisms changes only after several months consuming a new diet (Hesslein *et al.*, 1993; MacAvoy *et al.*, 2001; Jardine *et al.*, 2004). As a result, an organism's isotopic concentration can be used to assess the contribution of various food sources to the diet, as long as the food types have different isotopic concentrations and the trophic shift between prey and consumer is known (DeNiro and Epstein, 1978; DeNiro and Epstein, 1981).

Discrimination factors depend on diet quality, tissue and consumer's individual characteristics such as age (Kelly, 2000). Firstly, discrimination is sensitive to dietary nitrogen and carbon concentration and to diet quality (McCutchan *et al.*, 2003; Pearson *et al.*, 2003; Vanderklift and Ponsard, 2003; Robbins *et al.*, 2005). Also, it is influenced by nitrogenous waste loss (DeNiro and Epstein 1981; Minagawa and Wada, 1984; Michener and Schell, 1994; Kelly, 2000; Vanderklift and Ponsard, 2003). Furthermore, tissues do not reflect the isotopic composition of food in the same way, due to dissimilarities in turnover rate and

biochemical pathways (Hobson and Clark, 1992a,b; Hobson *et al.*, 1996; Roth and Hobson, 2000; Kurle and Worthy, 2002; Pearson *et al.*, 2003; Vanderklift and Ponsard, 2003).

The isotopic discrimination between tissues has been studied mainly in mammals (Hobson *et al.*, 1996; Roth and Hobson, 2000; Kurle and Worthy, 2002; Vanderklift and Ponsard, 2003) and birds (Hobson and Clark, 1992a,b; Thompson and Furness, 1995; Vanderklift and Ponsard, 2003), although it is still poorly known. Information about tissue discrimination in fish (Hesslein *et al.*, 1993; MacAvoy *et al.*, 2001; Logan *et al.*, 2006) and reptiles (Godley *et al.*, 1998; Struck *et al.*, 2002; Biasatti, 2004; Seminoff *et al.*, 2006) is even scarcer. The aims of this paper are (1) to describe how nitrogen and carbon isotopic concentration varies among tissues in loggerhead sea turtles (*Caretta caretta*) and (2) to assess the position of loggerhead sea turtles in food webs of the Mediterranean on the basis of their isotopic concentration.

## MATERIAL AND METHODS

The loggerhead sea turtles used in this study were collected off the Balearic Archipelago (western Mediterranean) from 2002 to 2004. Stranded individuals from Majorca and Minorca were sampled by the staff of the *Conselleria de Medi Ambient* and the *Fundación AsproNatura*. The straight carapace length (SCL) and the curved carapace length (CCL) were measured in each individual. In addition, the cause of death (interaction with drifting long-lines, interaction with trammel nets, collision with boats or other causes) was assessed, and samples of skin, muscle and carapace scute were taken. The staff of *Fundación AsproNatura* also took samples of skin, carapace scute and blood from live turtles found off these islands. Muscle samples were always collected from the proximal part of the right fore flipper, carapace scute samples from the central part of the dorsal and marginal scutes and skin samples from the distal tip of the right rear flipper. The *Vellmarí* marine animal rescue centre collected the same samples from live turtles around the Pitiusas Islands (Ibiza and Formentera). All samples were stored frozen (-20°C) until analysis.

After being dried at 60°C, samples were ground to a fine powder, and lipids were extracted from all tissues with a chloroform/methanol (2:1) solution.

They were then weighed into tin cups, combusted at 1000°C, and analysed in a continuous flow isotope ratio mass spectrometer (Flash 1112 IRMS Delta C Series EA Thermo Finnigan). Atropine was used as a system check for elemental analyses. Stable isotope abundances were expressed in  $\delta$  notation according to the following equation:

$$\delta X = ((R_{\text{sample}}/R_{\text{standard}}) - 1) \times 1000$$

where X is  $^{13}\text{C}$  or  $^{15}\text{N}$  and  $R_{\text{sample}}$  and  $R_{\text{standard}}$  are the corresponding  $^{13}\text{C}/^{12}\text{C}$  or  $^{15}\text{N}/^{14}\text{N}$  ratio of the sample and the standard. The standards for  $^{13}\text{C}$  and  $^{15}\text{N}$  were the Vienna Pee Dee Belemnite (VPDB) and atmospheric nitrogen (air), respectively. International isotope secondary standards for carbon (IAEA  $\text{CH}_6$  ( $\delta^{13}\text{C} = -10.4\text{‰}$ ), USGS 24 ( $\delta^{13}\text{C} = -16.1\text{‰}$ ), IAEA  $\text{CH}_7$  ( $\delta^{13}\text{C} = -31.8\text{‰}$ )) were used to a precision of  $0.2\text{‰}$  and standards for nitrogen (IAEA  $\text{NO}_3$  ( $\delta^{15}\text{N} = +4.7\text{‰}$ ), IAEA  $\text{N}_2$  ( $\delta^{15}\text{N} = +20.3\text{‰}$ ), IAEA  $\text{N}_1$  ( $\delta^{15}\text{N} = +0.4\text{‰}$ )) were used to a precision of  $0.3\text{‰}$ .

Turtles were classified into three groups according to their origin: individuals caught in drifting long-lines (all dead), individuals caught in trammel nets (all dead), and individuals hand-caught in the open sea (all alive). This classification was thought to be relevant as trammel nets usually catch turtles close to the sea floor in inshore waters, whereas drifting long-lines only catch turtles that spend most of their time in the offshore water column. Benthic and pelagic ecosystems differ in their stable isotope baselines and hence these two groups of turtles have to be analysed independently. Hand-caught turtles were collected on the continental shelf. Consequently, they may have access to the seabed. If this is the case, they belong to the same category as individuals caught in trammel nets. However, as there is no evidence that they actually behave in this way, they were kept in a separate category.

Normality and homoscedasticity of the data were tested with Lilliefors' and Levene's tests, respectively. The Hotelling's t-test was used to establish whether statistically significant differences existed between the isotopic concentration of samples from the dorsal and marginal scutes of the same turtle. The existence of statistically significant differences between the isotopic concentration of different tissues was tested by means of Multivariate Analysis of Variance (MANOVA), carried out independently on the three groups of turtles (long-line, trammel net and hand-caught turtles). When statistically signifi-

cant differences between tissues were observed, one-way analysis of variance (ANOVA) was performed, followed by the Tukey test to assess which of the two parameters ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) contributed to the differences. The existence of differences in the isotopic concentration of the skin and the carapace scutes of the three groups of turtles was assessed by means of multivariate analysis of variance (MANOVA). Moreover, differences in the isotopic concentration of the muscle of the turtles caught in trammel nets and in drifting long-lines were assessed by means of Hotelling's t-test.

As the trophic level of the loggerhead sea turtles increases with age (Godley *et al.*, 1998), one-way ANOVA was used to determine whether the three considered groups of turtles differed in their average SCL. In some cases, carapace length was measured using only one of the two systems established in the sampling protocol (CCL and SCL). As SCL is preferred (Bolten, 2000), CCL data were transformed to SCL using a linear equation calculated from a database including information from about 47 specimens stranded or caught off the Balearic Archipelago ( $\text{SCL} = 0.924\text{CCL} - 0.323$ ,  $r^2 = 0.970$ ,  $p < 0.001$ ).

The loggerhead sea turtles found off the Balearic Archipelago make ample use of the Algerian Basin (Cardona *et al.*, 2005) and hence values of the isotopic concentration of carbon and nitrogen reported for several species from the western and eastern limits of the Algerian Basin (Dauby, 1989; Dauby *et al.*, 1990; Jennings *et al.*, 1997; Lepoint *et al.*, 2000; Pinnegar and Polunin, 2000; Polunin *et al.*, 2001) were used to assess the trophic level of loggerhead sea turtles in the food web.

## RESULTS

In total, we analysed 128 tissue samples from 53 loggerhead sea turtles (Table 1). However, Hotelling's t-test revealed that the bivariate isotopic concentration of dorsal and marginal scutes from the same specimen did not differ ( $F = 2.036$ ,  $df = 2$ ,  $p = 0.142$ ,  $n = 25$ ), and consequently only one scute sample from each turtle was used for further analyses.

The bivariate isotopic concentration of the tissues analysed in each group of turtles is shown in Figure 1. The data did not depart significantly from normality and variance was homogenous in many cases, although the  $\delta^{15}\text{N}$  variance of the tissues from

TABLE 1. – The number of samples analysed from each tissue and turtle group according to method of capture. (\*): Scute samples whose origin (dorsal or marginal scute) was unknown.

	Drifting long-line	Trammel net	Caught by hand
Scute*	0	3	9
Dorsal scutes	10	8	7
Marginal scutes	10	8	7
Blood	0	0	27
Skin	8	8	7
Muscle	8	8	0

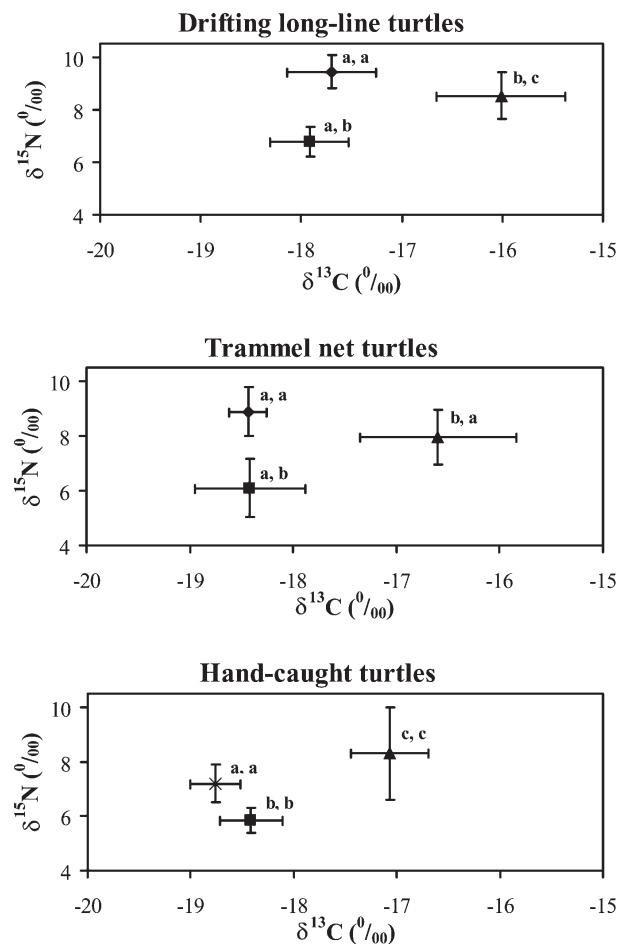


FIG. 1. – Isotopic concentration (mean ± standard deviation) of carapace scutes (■), muscle (◆), skin (▲) and blood (×) of turtles caught by drifting long-line (top panel), trammel net (center panel) and hand-caught (bottom panel). The tissues in each panel sharing the first upper index do not differ in δ¹³C, and those sharing the second one do not differ in δ¹⁵N.

the hand-caught turtles departed slightly from homoscedasticity. Nevertheless, this is a minor shortcoming (Scheiner, 2001). MANOVA revealed significant differences between the bivariate isotopic concentration of the different tissues analysed in the three groups of turtles (Drifting long-line: Pillai's trace=39.190,  $p<0.001$ ; Trammel net: Pillai's

trace=27.671,  $p<0.001$ ; Hand-caught: Pillai's trace=39.527,  $p<0.001$ ). One-way ANOVA showed that differences were due to both δ¹³C (Drifting long-line:  $F=39.687$ ,  $p<0.001$ ; Trammel net:  $F=19.770$ ,  $p<0.001$ ; Hand-caught:  $F=98.117$ ,  $p<0.001$ ) and δ¹⁵N (Drifting long-line:  $F=32.980$ ,  $p<0.001$ ; Trammel net:  $F=31.691$ ,  $p<0.001$ ; Hand-caught:  $F=24.641$ ,  $p<0.001$ ). A *post hoc* Tukey test revealed significant differences between the δ¹³C of the skin, scute and blood of hand-caught turtles (Fig. 1). The δ¹³C of the skin and scute were also statistically different in the other two groups of turtles. However, the δ¹³C of muscle did not differ from that of scute, but was statistically different from that of skin. Likewise, a *post hoc* Tukey test revealed statistically significant differences in the δ¹⁵N of skin, scute and blood of the hand-caught turtles. The δ¹⁵N of the three tissues (skin, scute and muscle) sampled from turtles caught in long-lines were also statistically different. The δ¹⁵N of the scute of turtles caught by trammel nets was significantly different from that of skin and muscle. However, differences between the δ¹⁵N of skin and muscle were not statistically significant in this turtle group.

When the isotope concentration of scute and skin was compared across the three groups of turtles, MANOVA revealed the existence of statistically significant differences for both tissues (Scute: Pillai's trace=3.710,  $p=0.009$ ; Skin: Pillai's trace=3.015,  $p=0.029$ ). One-way ANOVA revealed that differences were due to the δ¹³C for both tissues (Scute:  $F=5.602$ ,  $p=0.008$ ; Skin:  $F=5.589$ ,  $p=0.012$ ), whereas the δ¹⁵N differences were statistically significant for scute ( $F=5.343$ ,  $p=0.010$ ) but not for skin ( $F=0.381$ ,  $p=0.688$ ). A *post hoc* Tukey test revealed significant differences between the δ¹³C of the scute of the turtles caught in drifting long-lines and those of the other two groups of turtles. Similarly, the skin from the hand-caught individuals and that from those caught in drifting long-lines differed in the average δ¹³C, but none of them differed from that of trammel net individuals. Differences between the δ¹⁵N of scute were found between hand-caught turtles and those caught in drifting long-lines, but not between the two former groups and the turtles caught in trammel nets. On the other hand, there was no statistically significant difference between the δ¹⁵N of the skin of the three groups of turtles.

Comparison of the bivariate isotopic concentration in the muscles of turtles caught in drifting long-lines and trammel nets revealed the existence of sta-



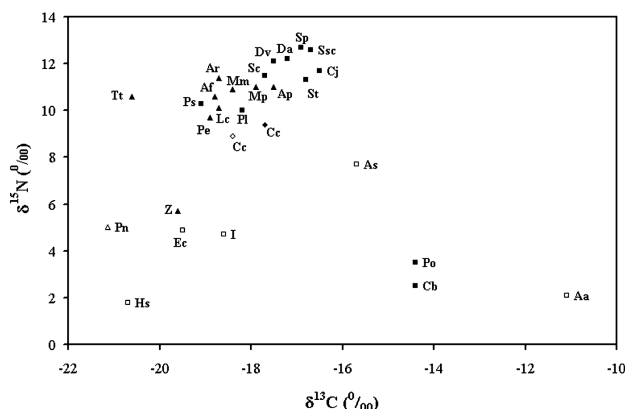


FIG. 2. —  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of several species: ( $\square$ ) benthic species from Corsica (*Acetabularia acetabulum* (Aa), *Halopteris scoparia* (Hs), *Ircinia* sp. (I), *Anemonia sulcata* (As), *Eunicella cavolinii* (Ec) (Lepoint *et al.*, 2000)), ( $\blacksquare$ ) benthic species from the Balearic Islands (*Scyllorhinus canicula* (Sc), *Pasiphaea sivado* (Ps), *Parapennaues longirostris* (Pl) (Polunin *et al.*, 2001), *Coris julis* (Cj), *Symphodus tinca* (St), *Diplodus annularis* (Da), *Diplodus vulgaris* (Dv), *Sarpa salpa* (Ssa), *Scorpaena porcus* (Sp), *Serranus scriba* (Ssc) (Jennings *et al.*, 1997)), ( $\triangle$ ) pelagic species from Corsica (*Pelagia noctiluca*), ( $\blacktriangle$ ) pelagic species from the Balearic Islands (Zooplankton (Z), *Alepocephalus rostratus* (Ar), *Lampanyctus crocodilus* (Lc), *Merluccius merluccius* (Mm), *Micromesistius poutassou* (Mp), *Trachurus trachurus* (Tt), *Aristeomorpha foliacea* (Af), *Plesionika edwardsi* (Pe) (Polunin *et al.*, 2001) and loggerhead sea turtles caught in trammel ( $\blacklozenge$ ) nets and in long-lines ( $\diamond$ ).

tistically significant differences (Hotelling's  $t=10.259$ ,  $p=0.002$ ). These differences were due to the  $\delta^{13}\text{C}$  (Student's  $t=4.665$ ,  $p<0.001$ ), but not to  $\delta^{15}\text{N}$  (Student's  $t=1.396$ ,  $p=0.184$ ).

Differences in the mean SCL of the three considered groups (Hand-caught:  $48.1\pm 11.5$  cm; Trammel net:  $48.7\pm 12.8$  cm; Drifting long-line:  $50.5\pm 5.6$  cm) were not statistically significant (one-way ANOVA,  $F=0.167$ ,  $p=0.847$ ).

Figure 2 shows the position of the loggerhead sea turtles in the trophic web of the Algerian Basin as revealed by the isotopic concentration of muscle. Muscle was chosen because it was the tissue analysed for most of the animals species included for reference (Dauby, 1989; Dauby *et al.*, 1990; Jennings *et al.*, 1997; Lepoint *et al.*, 2000; Pinnegar and Polunin, 2000; Polunin *et al.*, 2001). As a consequence, hand-caught turtles are not shown in the figure, but this is not relevant as previous analyses revealed no difference between hand-caught and trammel net turtles for the other tissues analysed. The  $\delta^{15}\text{N}$  of the loggerhead sea turtles was intermediate between those of zooplanktivorous cnidarians (*Anemonia sulcata*, *Eunicella cavolinii* and *Pelagia noctiluca*) and those of zooplanktivorous shrimps (*Parapennaues longirostris*, *Pasiphaea sivado* and *Plesionika edwardsi*) and zooplanktivorous fish

(*Lampanyctus crocodilus* and *Trachurus trachurus*). The  $\delta^{13}\text{C}$  values of the two groups of turtles shown in Figure 2 overlapped with those of pelagic species.

## DISCUSSION

The best way to assess isotopic discrimination among different tissues is to keep animals in captivity, feed them experimental diets for long periods and allow tissues to achieve equilibrium with them (DeNiro and Epstein, 1978; DeNiro and Epstein, 1981; Hobson and Clark, 1992a,b; Hobson *et al.*, 1996; Roth and Hobson, 2000). This is not difficult with mammals and birds, as high metabolic rates allow them to achieve diet-tissue equilibrium after a few months. However, achieving diet-tissue equilibrium in ectothermic vertebrates requires more prolonged captivity, due to lower metabolic and turnover rates, and the isotopic change is usually attributed to growth and not to metabolic turnover (Hesslein *et al.*, 1993; Herzka and Holt, 2000; MacAvoy *et al.*, 2001; Maruyama *et al.*, 2001; Logan *et al.*, 2006). This probably explains why only one captivity experiment dealing with reptiles has been published to date (Seminoff *et al.*, 2006).

Interpreting differences in the concentration of stable isotopes between tissues of wild animals might be obscured if animals have not achieved equilibrium with the diet, for instance because they alternate prey with different isotopic concentrations. The situation may worsen if the species shift prey on a seasonal basis, as tissues differing in turnover rate will exhibit different isotopic concentrations not only for physiological reasons, but also because they integrate different time scales. Furthermore, the isotopic concentration in dead animals might change due to microbial activity. However, laboratory experiments have revealed that the skin and blood of green sea turtles (*Chelonia mydas*) achieved isotopic equilibrium with diet only after 371 days (Seminoff *et al.*, 2006), thus indicating that modifications of the average isotopic concentration of a population due to seasonal changes in diet is unlikely in sea turtles. Furthermore, captive raised green sea turtles (Seminoff *et al.*, 2006) and wild loggerhead sea turtles (this study) exhibited a similar pattern of isotopic discrimination between skin and blood, as the former always showed higher  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values than the latter. Finally, dead and live turtles from the same habitat did not differ in their isotopic concentration,

as trammel net and hand-caught turtles, both from the continental shelf, did not differ although the former were always dead and the second always alive. As a consequence, we are confident that the pattern of isotopic discrimination observed in the wild loggerhead sea turtles studied here is not obscured by any of the above reported confounding factors.

Loggerhead sea turtles consume a wide array of prey, including jellyfish, cephalopods and fish (Bjorndal, 1997; Houghton *et al.*, 2000; Tomás *et al.*, 2001; Bentivegna *et al.*, 2003). Fish have been suggested to be a relevant component of the species' diet in the western Mediterranean (Tomás *et al.*, 2001), but the trophic level of the loggerhead sea turtles found off the Balearic Archipelago, as revealed by the  $\delta^{15}\text{N}$  (Peterson and Fry, 1987), is much lower than that of typical ichthyophagous species from the same area (Lepoint *et al.*, 2000; Jennings *et al.*, 1997; Pinnegar and Polunin, 2000; Polunin, 2001). Indeed, the trophic level of the loggerhead sea turtles is higher than that of zooplanktophagous cnidarians but lower than that of zooplanktophagous fish, thus revealing a diet based on the former group, although some species from higher trophic levels might be consumed. This hypothesis is consistent with the  $\delta^{13}\text{C}$  values reported for the pelagic carnivorous cnidarian *Cotylorhiza tuberculata* (Dauby, 1989).

$\delta^{13}\text{C}$  values are often used to infer habitat use, as benthic species are typically more enriched in  $^{13}\text{C}$  than pelagic ones (Fry and Sherr, 1984; France, 1995a,b). However, the  $\delta^{13}\text{C}$  is rather variable in the groups analysed to date in the Algerian Basin, either phytoplankton (Dauby *et al.*, 1990), benthic macrophytes (Dauby, 1989; Jennings *et al.*, 1997; Lepoint *et al.*, 2000) or zooplanktophagous fish and shrimps (Polunin *et al.*, 2000). The three considered groups of turtles differed in the  $\delta^{13}\text{C}$  of some tissues, but these differences were not always consistent. Furthermore, the  $\delta^{13}\text{C}$  values of the loggerhead sea turtles overlapped with those of pelagic animals and the range of variability was within that of other groups of pelagic species in the Algerian Basin. Thus, we conclude that differences in  $\delta^{13}\text{C}$  were spurious.

In conclusion, there was a consistent pattern in the differences observed in the isotopic concentration of several tissues between groups of wild loggerhead sea turtles and the trophic level of this species in the Algerian Basin is intermediate between that of carnivorous cnidarians and zooplanktophagous fish and crustaceans.

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